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(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US).

(72) Inventors: SOLLINGER, Hans, W.; 1202 Sherman Avenue, Madison, WI 53703 (US). KENEALY, William, R.; 2344 West Lawn Avenue, Madison, WI 53711 (US). KNECHTLE, Stuart, J.; 2268 Caine Road, Oregon, WI 53575 (US). BURLINGHAM, William, J.; 760 Forsythia Place, Madison, WI 53705 (US).

(74) Agent: BARTA, Kent; Michael Best & Friedrich LLP, 100 East Wisconsin Avenue, Milwaukee, WI 53202–4108 (US).

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(54) Title: INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

(57) Abstract

A novel therapy for immunosuppression utilizes an immunotoxin to destroy the entire population of allograft reactive T cells during or after a tissue transplant procedure, followed by natural reconstitution of a tolerized T cell population. The therapy leads to permanent tolerance of the allograft thereby obviating the need for maintenance immunosuppressive drug therapy.

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INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

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FIELD OF THE INVENTION

The therapy of the present invention relates to the field of transplantation, and more particularly, to a strategy for suppressing the immune response to an allo- or xenograft, and then reconstituting the immune system without further immunosuppression. The reconstituted immune system is made tolerant to the presence of foreign antigens, so that continuous immunosuppressive drug therapy is not required.

BACKGROUND OF THE INVENTION

In the field of organ transplantation, implantation of donor tissue by allograft or xenograft results in a cellular response leading to rejection. The cells recognizing the histocompatibility antigens of the graft belong to the class of T lymphocytes, although other cell types are implicated physiologically in the rejection process itself. It has long been known that transplant patients require immunosuppressive therapy to maintain healthy functioning of the graft. The goal of this therapy is to suppress T cell function just enough to block graft rejection, but maintain immunity against infection.

Several drugs have become available which selectively suppress lymphocyte function without appreciable toxicity and associated side effects in a majority of patients. Typically, the degree of success

expected, and the number and severity of side effects is reflected in the closeness of match in histocompatibility between host and donor tissue. In some patients in which the tissue match is nearly perfect, with only a mismatch at very minor histocompatibility loci, a low dose of drugs is indicated. These patients may have a slight depression in peripheral white cell counts (down to 5.0 to 5.5/ml compared to 6.0 to 6.5/ml), but will not display the joint pain, swelling, and other symptoms of a more significant mismatch or resulting from the immunosuppression therapy itself.

Usually more than one drug is prescribed to obtain the desired level of immunosuppression. The patient is then monitored at least monthly to ascertain that a suitable balance of drug concentrations is maintained, and to check other parameters such as hematocrit, serum creatine, white blood cell count, etc. Initially, large, tissue-saturating doses of these drugs are given, and the doses are then tapered off, until the patient's condition stabilizes at an optimal dose. Since rejection is mediated by infiltration of the allograft with inflammatory cells, an anti-inflammatory drug such as prednisone may also be given in addition to the immunosuppressive agent or agents. For a review of immunosuppressive drug monitoring combinational strategies, see Rugstad, "Immunosuppressive Drugs", in *Immunopharmacology in Autoimmune Diseases and Transplantation*, eds. Rugstad, et al., Chapter 18, p. 233 et seq., Plenum Press, N.Y.: 1992.

The most commonly used immunosuppressive drugs are Cyclosporin A (CyA) and thiopurines such as azathioprine (AZA). Lesser immunosuppressive drugs are: mercaptopurine, methotrexate, alkylating agents, and glucocorticoids. CyA, marketed under the tradename Sandimmune®, targets T-cells, in particular the helper T-cell population which are reversibly inhibited. It is not cytotoxic at therapeutic levels, does not

disrupt DNA synthesis, and does not give rise to lymphopenia. A major apparent impact is inhibition of lymphokine production by preventing the cytosolic components of transcription binding factors NF-AT and NF-kappaB from translocating to the nucleus, thereby inhibiting IL-2 gene transcription. It should be noted that CyA does not act upon mature cytotoxic T lymphocytes which are already differentiated, so that inhibition of lymphokine production may lead to inflammation.

Drugs, like CyA, with pronounced impact on normal cell function can be expected to produce adverse events. Among these are renal dysfunction such as reduced renal plasma flow and glomerular filtration rate. At higher doses, elevation in serum creatine and histologic lesions in the kidney are noted. CyA nephropathy has been described involving arteriolopathy, acellular interstitial fibrosis, and tubular atrophy.

The thiopurines, analogues of hypoxanthine, are mildly toxic resulting in bone marrow aplasia and hepatitis. Azathioprine, marketed as Imuran®, is active as a metabolite, thioinosinic acid. While the drug may cause some suppression of macrophage mediated delayed hypersensitivity, its action is primarily directed to T-cell dependent immune responses, especially at low doses. This preferential effect on T cells, with only slight inhibition of B cell responses (possibly attributable in part to helper T cell inhibition) accounts for its clinical popularity. The corticosteroids can cause aseptic bone necrosis, cataracts, and diabetes.

A very interesting group of immunosuppressants are anti-T-cell monoclonal antibodies, which act either by cell destruction or inactivation (receptor blockade), or by immune modulation in which there is a redistribution and disappearance of the target molecule from the cell surface. One such antibody studied in

the murine model is anti-L3T4, similar to the corresponding antibody to the CD4 marker. Injection of anti-L3T4 results in depletion of over 90 percent of L3T4+ cells resulting in complete suppression of humoral responses. Treatment with this antibody also significantly extends allograft survival, but does not result in tolerance (permanent non-recognition of the allograft).

In primate models, it has been found that monoclonal antibodies against the CD4+ and CD8+ T cell subsets were immunosuppressive in monkeys receiving renal or skin transplants. The mechanism appears to be immune modulation, since there was no evidence of CD4+ cell depletion in animals treated only with OKT4 antibody. There was immunosuppression, in contrast to studies in which only anti-CD8 antibodies were injected. For an overview of the effects of monoclonal antibodies directed to various lymphocyte markers having a known or suspected role in allograft rejection, see Chattenoud, et al., "Anti-T-cell Monoclonal Antibodies as Immunosuppressive Agents", in *Immunopharmacology in Autoimmune Diseases and Transplantation*, supra, Chapter 14, p. 189 et seq.

Another interesting reagent, licensed for immunosuppression therapy, is OKT3 antibody. OKT3 is a pan-T-cell antibody capable of recognizing an antigen on essentially all T cells. In conjunction with low doses of other immunosuppressive agents, OKT3 reverses acute allograft rejection in renal transplantation. Prolonged administration of OKT3 results in complete depletion of CD3+ cells so long as a detectable level of OKT3 can be maintained in peripheral blood. Eventually, there is a reappearance of CD3-CD4+ and CD3-CD4+ antigen modulated cells; but no allograft rejection occurs in the absence of CD3+ T cells.

The use of OKT3 and other anti-pan-T-cell antibodies have the disadvantage of inducing sensitization to the murine determinants. Once anti-

OKT3 antibodies appear, there is a rapid elimination of the agent from peripheral blood, thereby abrogating its therapeutic value. In experiments with mice rendered tolerant to rat species specific determinants, the mice nevertheless developed high titers of anti-idiotypic antibodies directed to the variable region. Thus, even humanized forms of these antibodies may induce immune responses if the variable complementarity directing regions are not present in the human repertoire.

All of the drug or biologics based therapies described above for immunosuppression have the disadvantage of requiring lifelong continuous treatment with a therapeutic regimen. Unless drug administration is carefully monitored, imbalances can lead to a spectrum of side effects. Too little suppression may provoke a rejection episode; too much suppression can cause leukopenia and other abnormalities of blood cell numbers and ratios. As transplant procedures become more routine, there is an increasing need for more complete and systemic immunosuppression mechanism.

SUMMARY OF THE INVENTION

In the present invention, permanent tolerance to an allograft or xenograft is achieved by selectively eliminating all T cell clones capable of mediating an immune response to the foreign cells resulting in acute rejection thereof. It is an object of the invention to achieve permanent tolerance to the allograft without the need for a transplant patient to take maintenance doses of immunosuppressive drugs to control the rejection process.

A second object is to provide a therapy in which a transient period of immunocompromise is short lived, and does not affect the immunoglobin-secreting B lymphocyte immunity already in place. A related aspect involves maintaining the T-cell pipeline fully

functional up to the last stages of maturation and differentiation.

A further object is to provide a relatively non-toxic reagent for inducing permanent tolerance to an allograft or xenograft administrable in one or a few bolus doses peritransplantationally.

The method of inducing immune tolerance to an allograft in a mammal comprises administering a therapeutically effective amount of an immunotoxin consisting of restrictocin molecularly conjugated to an monoclonal antibody having CD3-like binding specificity. This method results in destruction of essentially all T cells capable of mediating immune rejections of transplanted tissue. The method is further characterized in preferentially destroying all such reactive T cells thereby depleting them, and then allowing repopulation of the immune system by natural regeneration of T cells in the absence of immunosuppressive agents.

The reagent of the present invention is a molecular conjugate which can induce permanent immune tolerance towards allogeneic cells in a recipient mammal comprising restrictocin covalently conjugated to an antibody through a linkage cleavable under physiological conditions within the target cells, and an antibody contained in the conjugate having OKT3 characteristics such as affinity, marker specificity, and toxin presentation and release properties.

The reagent is contained in an article of manufacture comprising a sterile container of the type used in the pharmaceutical art to contain an injectable solution under pharmaceutical conditions of purity and sterility, a solution of the reagent antibody-toxin conjugate having the requisite antigen recognition specificity corresponding to OKT3, and a label affixed to the container bearing directions for administration of the reagent in a dose sufficient to destroy essentially all peripheral T cells outside of the bone

marrow to thymus pathway, including all those T cells having allograft rejection capability.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing the effect of restrictocin on various mitogen stimulated proliferation challenges of target T cells. Also shown are data for mixed lymphocyte reactions.

Figure 2 is a rectilinear plot of the effect of OKT3-restrictocin on mixed leukocyte proliferation at various doses.

Figure 3 is a graph which plots the average percentage of CD4, CD8 and $CD45^+$ positive cells in the peripheral circulatory system against time expired since PBL injection.

Figure 4 is a graph which plots the average percentage of CD4, CD8 and $CD45^+$ positive cells in the peripheral circulatory system and in the spleen against time expired since PBL injection.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In a mammal, cellular immune responses are mediated by T cell populations that recognize the antigenic determinants present on transplanted allografts. T cells are so named because they represent a subpopulation of lymphocytes which originate in the bone marrow and migrate through the thymus gland where they mature into various subclasses of cells. This maturation is a generally continuous process, with continual regeneration of T cell subpopulations from bone marrow derived stem cells. The ontogeny of T cells is a very complex process, as yet not well understood. However, it is believed that

anti-self clones are eliminated in the thymus epithelium by clonal deletion involving an abortive interaction of various cell surface molecules including CD3, CD4, CD8, one of the class of histocompatibility antigens, and the T cell antigen receptor.

In acquired tolerance, both clonal deletion models and clonal anergy have been demonstrated. Clonal anergy is a state of down regulation of the clones, which now become inactive in the presence of antigen. The factors generally recognized to play a role in tolerance induction are the immune competence of the host, the structure and configuration of the antigen, the immunogenicity of the antigen, the route of administration, and the dose. In high zone tolerance very large amounts of antigen induce a short-lived paralysis of the immune response. Low zone tolerance occurs upon administration of low doses of T cell dependent antigens, is rapidly established, and relatively long-lived. Clonal deletion models assume that apoptosis of the emergent clone is achieved in the epithelial microenvironment of the thymus, and that anergy occurs in the peripheral lymph system after T cell maturation has already occurred. Precisely what mechanisms are involved in allograft tolerance induced after massive depletion of the native immune system, as in whole body irradiation is not completely known.

Reconstitution of the immune system with autologous stem cells or donor allogeneic cells after destruction of the native lymphocytes has been applied in many situations involving blood cell neoplasms. (For a review, see Fritz Bach, et al., *Transplantation Immunology*, Wiley-Liss Inc.: 1995). Frequent complications include graft versus host reactions, and immunocompromised immune systems. This strategy has been very effective in murine models to induce tolerance of the allogeneic cells by eliminating all the cells capable of recognizing the antigenic determinants. In one interesting study, transgenic

mice expressing a cell membrane egg lysozyme were mated to a second transgenic strain carrying a particular anti-lysozyme IgM gene. B lymphocytes carrying the IgM gene could not be detected, suggesting clonal deletion.

In the method of the present invention, tolerance to an allograft (and potentially a xenograft) is obtained by first destroying all T cells capable of mediating an anti-allograft response, without killing the regenerative source of stem cells in the bone marrow. Newly regenerated stem cells will then migrate to the thymus where T cell maturation takes place. Because the allograft is now part of the body, the immune system will perceive the allograft as self and the reactive clones will either be depleted or anergized.

One of the Applicants hereof has recently demonstrated the feasibility of this approach in a rhesus monkey model in which renal allograft tolerance was induced by prior treatment with a diphtheria-derived toxin conjugated anti-T cell antibody (see Knechtle, et al., *Transplantation*, 63: 1 (1997). Several of the animals pretreated with the toxin-conjugated antibody alone showed long term survival without acute rejection, whereas all of the untreated animals experienced acute rejection in 7-10 days. The system described in this paper may be of limited use in humans because the nearly universal presence of neutralizing antibodies to the diphtheria-derived toxin would quickly eliminate the therapeutic agent in immunized populations. Also, the immunotoxin was not uniformly effective, as some treated animals eventually rejected their allografts, suggesting that some reactive cells were spared in the pretreatment. In order to mitigate the toxicity of the diphtheria-derived toxin, a mutant form having an altered binding site was used. While still possessing potent protein synthesis disrupting activity, it may be sufficiently attenuated to permit some reactive cells to escape.

Clearly, the completeness of T cell destruction is critical to the efficacy of this approach to achieving permanent tolerance to the allograft.

The immunotoxin of the present invention utilizes the toxin restrictocin molecularly conjugated to an antibody having the specificity of OKT3. OKT3 is a monoclonal antibody disclosed and claimed in U.S. Patent No. 4,361,549 hereby incorporated by reference, and developed by Ortho Pharmaceutical Corporation. It is preferred in the present invention for two reasons. First, it has the requisite specificity, recognizing essentially all differentiated T cells expected to be reactive to allogeneic cells. Secondly, it has already passed the scrutiny of the U.S. Food & Drug Administration. The antibody is licensed under the tradename Orthoclone OKT3 for use as an immunosuppressant in conventional therapy (see Physicians' Desk Reference, 1996, p. 1837 et seq.).

The power of monoclonal antibody technology is that every antibody molecule in the total clonal output has exactly the same primary structure, and therefore the same specificity for a single epitope. In the case of OKT3, specificity is directed to the CD3 epitope. This is an extremely interesting marker in the present application as it is believed that T cells only express this marker just at the point where the T cells are close to full differentiation and release from the thymus. This is important because it is desirable to clonally delete or suppress the allograft reactive cells, while at the same time, immunocompromising the patient to a minimal extent.

During the course of the present therapy, there will a short period of time in which the body will be somewhat vulnerable to challenge by pathogens having T cell dependent antigens. However, since essentially only peripheral T cells will be affected, there will be no disruption of the normal development of new T cells from bone marrow to the thymus. This means that

restoration of complete immunocompetence will require less time and involve less risk than if an early T cell marker such as CD1 were selected. Since antibodies having OKT3 specificity recognize only T cells and not B cells, the ongoing and memory induced humoral responses will not be affected by this therapy.

However, the antibody of choice in the present invention is not limited to OKT3, but may be any monoclonal antibody with substantially the same specificity of OKT3. Such an antibody is herein sometimes referred to as OKT3-like. By the term "same specificity" Applicants mean an antibody having an antigen binding specificity which recognizes the same cell surface marker as OKT3, whether or not precisely the same epitope, and which has sufficient binding affinity, generally in the nanomolar (nM) range, recognize and bind target T cells and discharge the immunotoxin. Therefore, the term also means any antibody having specificity for CD3 which presents the toxin so that it is taken up into the cytoplasm.

The antibody may be of any class suitable to this indication, preferably class IgG1. It may be a recombinant form altered as to its species specific components to become humanized (e.g. nonimmunogenic to humans). The antibody may also be a stabilized, truncated form, a single chain antibody, or conventional enzymatically prepared fragments, such as Fab, known in the art. These smaller forms may be particularly preferred where non-specific tissue interactions involving the Fc region are to be avoided, and where the smaller forms may diffuse more readily into the sites of T cells and T cell clones resident in solid tissue. The preferred toxin in the present invention is restrictocin, a fungal toxin secreted by *Aspergillus restrictus*. Restrictocin is one of a class of fungal toxins which have been evaluated as conjugates for delivery to target cells in therapeutic strategies. For an older, but informative overview of

the field of plant and fungal proteins and glycoprotein toxins affecting eukaryote protein synthesis, see Jimenez, et al., *Ann. Rev. Microbiol.*, 39: 649 (1985). Of the four most studied fungal toxins, alpha-sarcin, mitogillin, aspf1, and restrictocin, restrictocin is preferred for the following reasons: (i) extremely high specificity and efficiency in cleaving 28s rRNA, (ii) availability in commercial quantities of native, recombinant, and mutant forms, (iii) apparent lower toxicity to non-targeted cells of toxin dissociated from its conjugate, and (4) ability to cross cell membranes (see, for example, Yang, et al., Structure, 4: 837 (1996)).

One reason for restrictocin's superiority as an immunotoxin is that the free molecule lacks a cell binding domain, and requires a binding intermediary such as an antibody, for entry into cells. Thus, an unprotected bond between antibody and restrictocin can be used to enhance cytoplasmic delivery without toxic side effects. These side effects are obviated since circulating free restrictocin released by physiologically mediated sulfhydryl reduction will not penetrate untargeted cells. Another reason for restrictocin's superiority is that stable conjugation requires only one linkage since restrictocin is a single chain protein of 149 amino acids. Another advantage to restrictocin is that it is a small molecule compared to other toxins, and therefore less immunogenic. A discussion of these advantages is set forth in Rathore, et al., *Biochem. and Biophy. Res. Comm.*, 222; 58 (1996).

Restrictocin may be obtained and purified by essentially the original published method, as modified. U.S. Patent No. 3,104,208, incorporated by reference, disclosed a method for producing and purifying restrictocin over 30 years ago. The organism (NRRL 2869) was grown in a rich undefined medium under specified conditions of temperature and aeration. The

supernatant fluid was applied to an Amberlite ICR-50 column, and the eluted restrictocin thus purified. Further purification utilizing Millipore Pellicon filtration was described by Yang, et al., *J. Gen. Microbiol.*, 138: 1421 (1992), hereby incorporated by reference. In the Example of this application, a further hollow fiber concentration step is followed by HPLC. There are now available methods known in the art of carrying out preparative HPLC or its equivalent for production of commercial quantities of restrictocin sufficiently pure to meet FDA QA/QC requirements.

Conjugation of restrictocin to OKT3-like antibody requires an intermediate step since the native molecule does not contain a free sulfhydryl group. Conde, et al., *Eur. J. Biochem*, 178: 802 (1989), hereby incorporated by reference, described the basic procedure for derivatizing restrictocin with N-succinimidyl 3-(2-pyridyldithio) proprionate (SPDP). The OKT3-like antibodies are similarly derivatized with the same agent. The purifications separating the derivatives from the unreacted species are also described in detail. It is also efficacious to generate sulfhydryl groups via 2-iminothiolane. Complete reaction specifications, reagents, and procedures for utilizing various derivatizing and conjugating strategies are set forth in G.T. Hermanson, *Bioconjugate Techniques*, Academic Press: 1996), hereby incorporated by reference. See especially Chapter 7, "Cleavable Reagent Systems", pp. 292 et seq. for a detailed description of conjugates which release the conjugated toxin. While emphasizing the use of cleavable systems in structure verification and moving a molecule from one conjugate to another, it is apparent from Conde, et al., supra, that these systems can be used to produce immunotoxins directed against human tumor cells.

In a preferred method of preparing and purifying the immunotoxin conjugate, the reacted material is

purified by double affinity chromatography, as disclosed in detail in the Examples. Restrictocin-specific antibodies are covalently bound to a column matrix. This column is then used to bind the restrictocin-conjugated OKT3-like antibodies. The recovered, eluted conjugate is then purified again on an affinity column which binds antibody. The final eluant may then be finally purified and the product confirmed by gel filtration. The double affinity procedure results in a more highly purified conjugate than conventional gel filtration alone. A relatively small column bed can be used for even commercial scale batches, since the bulk of material passes through the column in a wash, and the amount of bound material is small, particularly in the second step.

One reason for refining the purification process for preparing the immunotoxin as much as possible is addressed in R.W. Baldwin, et al., eds., *Monoclonal Antibodies and Immunoconjugates*, Parthenon Publishing Group: 1990. In the tumor field it was found that the presence of free antibody effectively competed for sites on tumor cells, thus sparing some cells from the lethal effect of the toxic conjugate. This led to much shorter survival prolongation times than expected. Improved purification procedures only marginally improved this result, since it was found that free antibody was generated readily from the natural dissociation of the disulfide bonds holding the conjugate together, under physiological conditions. The article goes on to teach that the problem can be overcome by utilizing another coupling agent N-succinimdyl-oxycarbonyl-alpha-methyl-alpha-(2-pyridyldithio)toluene (SMPT). The disulfide bond in the conjugated is protected from thiol attack by the presence of an adjacent methyl group and benzene ring. The structures of these and other coupling agents useful in biological applications are given in *Double-*

Agents, Cross-linking Reagents, A Selection Guide, published by Pierce Chemical Company, 1996.

The prior discussion here of the problems of adapting immunotoxins to the field of oncology emphasizes the contrast to the requirements of tolerance induction in the field of transplantation. Contrary to the above teaching, it is an advantage to use an unprotected disulfide link between the toxin and antibody, because the ease of dissociation under particularly intracellular conditions helps to ensure complete penetration of all target T cells with high efficiency. As set forth above, the premature release of free restrictocin in small amounts will not produce prohibitively debilitating side effects, as with alpha-sarcin, because it is potent in lower levels generally, and the free restrictocin has no cellular binding domain.

In the therapeutic method of the present invention, the restrictocin-OKT-like purified conjugate is administered, preferably by intravenous injection, in a therapeutically effective amount. The blood levels of free restrictocin, and the conjugate can be monitored by conventional assays, and the blood level of the conjugate maintained at a threshold level of .01 to 15 ng/ml, and generally in a range empirically determined to result in complete tolerance to the transplant. Thus, a therapeutically effective amount is that level of the conjugate which saturates the peripheral blood and tissues.

The administration of the total amount may be a bolus given at one or several times, or a continuous IV administration peritransplantation, beginning prior to the actual transplant, either accompanied or not by intrathymus infusion of the donor cells or an extract thereof, and continuing for several days thereafter. The amount given should at least be sufficient to prevent emergence from the thymus of new differentiated T cells during treatment. In general, the treatment

regimen for use of OKT3 in immunosuppression may be a guide. This regimen consists of a 5 mg bolus dose administered on each of 10 to 14 days post rejection episode.

Another advantage of the present method is that it may be used with patients who have already undergone transplant, and are repelling allograft rejection by maintenance doses of conventional immunosuppressive drugs. So long as there is a continuous source of allogeneic cells, and the dose administered peritransplantally, of restrictocin-OKT3 conjugate is therapeutically effective as herein defined, there is no distinction as to the timing of the allograft or xenograft, whether it be substantially simultaneous, or occurring at some remote time.

Further advantages of the present invention will be apparent from the Examples which follow.

EXAMPLE 1

Production of Restrictocin.

Restrictocin is obtained from the growth media of the fungus *Aspergillus restrictus* propagated in a liquid medium fermenter. The medium can be minimal or complex, with the important feature that the fungus must be cultivated for a sufficient period of time that it will undergo conidiation in solution.

There should be sufficient nutrient that it will continue to metabolize after it has undergone most of its growth and begun to exhaust some of the nutrient in the medium. An important aspect of minimal growth on acetate and ammonium as the carbon and nitrogen sources is to control the pH at 7.6 where the conidiation seems best and growth can continue. The production of restrictocin can be monitored via HPLC or by immunoblots, as described in Brandhorst, Yang and Kenealy, "Heterologous Expression of the Cytotoxin Restrictocin in Aspergillus Nidulans and Aspergillus

Niger", *Protein Expression and Purification*, 5, 1994, 486-497.

EXAMPLE 2

Purification of Restrictocin.

The hyphal material is separated from the culture filtrate by coarse filtration through Whatman filters or cheesecloth, miracloth or the like. The hyphal material is washed to remove residual restrictocin and then the filtrates pooled. The following steps can be done in any order.

Cation exchange chromatography. The filtrate is applied to the column (no adjustment in pH if between pH 5.0 and 8.0). The salt content of the medium should be below that of 0.75M NaCl or its equivalent in ionic strength. The preferred resin, Amberlite IRC-50, is described in U.S. patent nos. 3,104,204, 3,104,208, and 3,230,153. The resin is prepared first by soaking in 1N NaOH (with constant mixing) followed by a rinse in distilled or deionized water followed by neutralization with dilute phosphoric acid until the suspending aqueous solution above the resin remains between pH 7.0 and 5.0. At this point the resin can be loaded into a column and rinsed with 10 columns volumes (or more) of distilled or deionized water.

Once the filtrate is applied to the column, the column is eluted with distilled water until the absorbance trace on a UV monitor decreases to baseline. The column is then eluted with 0.5 M NaCl followed by a gradient or step elution to a concentration of 5 M NaCl. The UV-absorbing material (280nm) is collected and peaks are pooled. The restrictocin containing peaks (determined via HPLC or immunoblots) are collected and subjected to Ultrafiltration.

Ultrafiltration. The pooled peaks from the column or the filtrate directly is filtered on an AGT 30,000 NMW hollow-fiber filter. The filtrate

(permeate) is collected and the retentate is washed several times with distilled water with the filtrate being pooled together. This procedure removes proteins of greater than 30,000 MW and the restrictocin should pass through the filter matrix. The hollow fiber cartridge is then cleaned with 0.5N NaOH and distilled water and stored in 30% ethanol at 0-10 C. If the filtration is done first the filtrate is then put onto the column above. If the filtration is done after the column chromatography then the filtrate is concentrated and diafiltered as below.

Concentration and diafiltration. This step is best done after the filtration above. It will be required eventually after the chromatography since there will be large amounts of salt in the solution. The hollow fiber cartridge AGT 3000 NMW is taken out of storage and the ethanol washed away with distilled water. The pump and holding tank are also well rinsed prior to use. The filter is used to concentrate the material and the concentration of salt in the solution is reduced by adding distilled water to the reservoir holding the retentate. This effectively retains the protein of interest and by determining the hold up volume and dilution the amount of salt present can be calculated and reduced in the final retained material. The filtrate from this material is usually discarded. When the amount of salt is considerably less than the amount of restrictocin the concentration is brought to its lowest volume and the material recovered from the filter and associated piping rinsed with distilled water. All the protein containing washes are pooled freeze-dried.

Reverse Phase HPLC. The material treated with all the steps above is then purified to homogeneity by C-4 reverse phase chromatography (1). The fractions from the chromatography are quickly brought to dryness using a cool rotary concentrator and then rehydrated using distilled water. If this is not done quickly enough

there will be some degradation of the protein and some will precipitate out of the solution. The trifluoroacetic acid and acetonitrile are removed in this drying step. The resuspended protein can have a small amount of buffer added to it to neutralize the acidic groups on the protein. This material can be frozen and stored or freeze-dried and stored until used further.

Activity testing. When the protein is pure an approximate extinction coefficient of A280=1.0 for a 1 mg/ml solution can be used to estimate the protein concentration. The material purified in the above manner is tested for its ability to generate the alpha-fragment from ribosome preparations, as described in Brandhorst and Kenealy, supra. This must be done to assure the activity of the protein is present and that the protein is active as a toxin.

EXAMPLE 3

Conjugation Methods.

Restrictocin does not contain any free sulfhydryls as purified. The four cysteines present in restrictocin are coupled together in two disulfide bonds. The conjugation methods thus far attempted have focused on using a disulfide link between the antibody and restrictocin to hold the molecules together. This is advantageous since the disulfide bond is one that is relatively easy to cleave inside cells. Thus the toxin and antibody are connected in a bond that is designed to come apart after the toxin is delivered to the cells. The antibodies made usually do not have free sulfhydryls present and both proteins have to be modified to form the desired bond.

For purposes of this synthesis the restrictocin is modified to have a free sulfhydryl and the antibody modified so that a sulfhydryl is available that is protected with a good leaving group. This will allow

the exchange of a free sulfhydryl for the leaving group and the bond between the antibody and restrictocin will be formed.

The methods used are similar to these outlined in Hermanson, G.T., *Bioconjugate Techniques*, Academic Press: San Diego, CA, 1996. Restrictocin is modified by adding SPDP which binds to free primary amines. Reactions are also planned for sulfhydryl generation via 2-iminothiolane. The molar ratio of restrictocin to SPDP is one to one which means that some restrictocin will not be modified and some will be modified twice. After this first attachment of SPDP the modified restrictocin is reduced with dithiothreitol and excess reactants are removed via filtration on a 3-5,000 NMW filter or via gel filtration to desalt the restrictocin (for 2-iminothiolane the reduction is not done since the reaction provides a free sulfhydryl). The buffer at this point is roughly neutral in pH and contains EDTA to inhibit metal catalyzed oxidations of the free sulfhydrls. The restrictocin preparation is washed on the filter with this buffer and then removed from the filter to estimate the concentration of restrictocin (280nm).

The antibody is modified in a similar manner except the molar ratio of SPDP in the initial reaction is 3 SPDP to 1 antibody. Also the reduction does not take place, instead the preparation is filtered using a 100,000 NMW filter and washing with the above EDTA containing buffer. At this point that antibody is removed from the filter and the concentration estimated. A molar ratio of 3-6 restrictocins to one antibody is mixed together and allowed to react. After sufficient time has elapsed the mixture is filtered on the 100,000 NMW filter and rinsed with the above buffer. This mixture contains a variety of forms of the proteins. There will be some unconjugated

restrictocin and antibody remaining along with several ratios of restrictocin to antibody conjugates.

EXAMPLE 4

Conjugate Purification.

The conjugate is purified by size, and by affinity chromatography. The filtration and washing on the 100,000 NMW filter, if done extensively, above can remove all of the unreacted restrictocin. However the best purification uses affinity chromatography for both the antibody and restrictocin. Antibodies that bind restrictocin are generated in rabbits or chickens, affinity purified using restrictocin bound to column and then the antibodies are eluted and conjugated to a second column. The antibodies will bind restrictocin and not the monoclonal antibody. So only the conjugates with restrictocin attached and any free restrictocin will bind to the column. The bound material is eluted from the column, the eluting agent neutralized either by filtration or drying and then the material is again affinity purified on a column that binds the antibody portion of the conjugate. Once the bound material is washed and eluted off the second affinity column the only compounds in solution should have restrictocin and antibody coupled together.

This material can be used as a test material or it can be further refined using a size fractionation which should separate the 1:1 2:1 etc. ratios of restrictocin to antibody from each other. The conjugates will also be analyzed by polyacrylamide gel electrophoresis in both a reducing form and non-reducing form. Reduced samples will show the subunits that go to make up the antibody and the amount of restrictocin present. The non-reducing samples will indicate the size of the whole conjugate.

EXAMPLE 5

The efficacy of the restrictocin-OKT3 was evaluated in a series of invitro tests designed to determine the completeness of blocking T cell responses to a wide range of T cell stimuli. Inhibition of proliferative response in mixed leukocyte tests were also carried out to determine effective invitro doses required for complete ablation. The tests were carried out according to the following protocol:

Human peripheral blood leukocytes were isolated by ficoll hypaque which separated the population of reactive T cells (and other white blood cells such as B lymphocytes and monocytes) from granulocytes and residual red blood cells. In certain experiments, non-tissue culture treated EIA plates were precoated with soluble antigens or mitogens for 24 hours prior to addition of responding cells (EIA plates, Costar # 3791). An identical plate was established in tissue culture-treated plates by adding antigen at the time of cell input (TC plates, Costar #3799). In other TC plates, mitogens and polyclonal stimuli were added to responder cells at optimal concentrations. After three days, all culture wells were harvested of 100 ul of media to test for production of cytokines, while the remaining cells were split into two plates; one to be assayed for viability by MTT dye technique, the other was pulsed with 3H-thymidine for 18 hours.

Detection of cytokine release was achieved by ELISA (Endogen and R & D matched pair kits), while MTT metabolism was performed by solubilizing the metabolized tetrazolium crystals in solubilization buffer. In both cases color differences were determined by microplate ELISA reader (Biotek). Thymidine incorporation was determined by harvesting cell contents, washing, a counting in a scintillation counter (Matrix 96, Packard Instruments).

The mixed lymphocyte reactions were run in two batches to test the concentrations required to suppress proliferation. The results are tabulated in Tables 1A and 1B. The results of batch 1 are also plotted in figure 2. It is apparent that the OKT3 antibody alone exerts some stimulatory effect on mixed cells at high concentrations. This effect is not completely abated until concentrations of antibody are reduced from 1000 ng/ml to less than 30 ng/ml. In contrast, concentrations of as little as 250 ng/ml of OKT3 conjugated to restrictocin are effective in preventing essentially any T cell stimulation, and some suppression is noted even at concentrations as low as 4 to 8 ng/ml.

In batch 2 (Table 1B), the effect of OKT3-restrictocin was compared at two levels of purity. A "crude" preparation was compared to one involving an extra column purification step. No differences were detected, indicating that the presence of small amounts of free reagent do not interfere with the suppressive effect under invitro conditions.

Figure 1 is a bar graph plotting a comparison of antigen and mitogen stimulation under conditions of treatment with OKT3-restrictocin, DAB-486, which is a highly toxic positive control consisting of a molecular construct of IL-2 and diphtheria toxin C chain, and an untreated control containing media only. The results clearly and dramatically show that OKT3-restrictocin inhibition of mitogen stimulated proliferation is essentially complete at the minimum inhibitory dose established in the batch experiments described above. The inhibitory effect was, in fact, more complete than for DAB-486, and was evident for all antigen/mitogens tested (from left to right, media only control, concanavalin A, High IL-2 (100 units), Poke Weed mitogen, Phabal myristate acetate, lipopolysaccharide, phytohemagglutinin, and the mixed lymphocyte reaction. This is expected to correlate with essentially complete

suppression and elimination of allograft reactive clones in the T cell population in vitro.

EFFECT OF OKT3-RESTRICTOCIN ON MLR PROLIFERATION

Table 1A

[CONC]	OKT3 mAB		T3-RESTRICT. BATCH 1	
(ng/ml)	MEAN	STD	MEAN	STD
1000	67.1	4.4	0.0	0.0
500	66.2	2.7	0.1	0.0
250	56.1	4.8	1.1	0.9
125	47.6	6.1	11.3	1.1
63	38.7	0.8	15.9	3.0
31	32.2	4.7	8.2	3.4
16	27.0	1.3	14.7	4.4
8	27.2	0.9	6.9	3.0
4	27.8	3.2	14.5	4.2
2	27.3	13.7	19.1	4.1
PBS VEH	25.2	3.6	21.5	3.7
UNTREAT	25.9	5.7	24.1	0.1

RESP = AL G. STIM = DEB MACK

Table 1B

[CONC] (ng/ml)	T3-RESTRICT. BATCH 2		[CONC] (%)	T3-RESTRICT. CRUDE	
80	8.6	1.0	20.0%	19.6	2.6
40	10.4	3.9	10.0%	27.5	0.5
20	17.6	3.8	5.0%	26.7	0.4
10	21.4	1.2	2.5%	21.2	0.2
5	19.1	2.2	1.25%	19.4	2.8
2.5	27.4	3.0	0.63%	21.6	0.6
1.25	19.2	3.0	0.31%	22.1	2.1
.63	22.3	5.0	0.16%	20.6	4.3
.31	26.3	5.2	0.08%	23.4	3.6
.16	33.8	1.2	0.04%	19.1	3.8
PBS VEH	32.8	5.1	0.02%	22.2	0.6
UNTREAT	27.6	8.7	UNTREAT	26.2	9.5

EXAMPLE 6

The efficacy of OKT-3 restrictocin in decreasing the number of human T-cells in vivo was examined. Briefly, on Day 1, SCID mice were injected with $1X10^8$ human peripheral blood lymphocytes (PBL). On day 8, a piece of human skin of full thickness was grafted onto the SCID mice. On Day 9, the mice were injected with 5μg OKT-3 restrictocin or with PBS as a negative control. At 3, 4 and 7 weeks post-PBL injection, flow cytometry was used to determine the extent of reconstitution of human CD4, CD8 and $CD45^+$ cells (T cells). The data are presented in Figures 3 and 4.

With respect to skin graft rejection, no difference was seen between control and treatment groups. This is probably because in this experimental system rejection of the human skin can be masked by extensive vascularization of the skin graft with mouse endothelium.

This experimental system allows the determination of the effect of treatment on T cell numbers. Figure 3 is a graph which plots the average percentage of CD4, CD8 and $CD45^+$ positive cells in the peripheral circulatory system against time expired since PBL injection (Im Tx- OKT-3 restrictocin treated SCID mice, NC- negative control PBS treated mice.) These data demonstrate that OKT-3 restrictocin treatment administered in only a single dose dramatically reduces the number of T cells in PBL injected SCID mice. The suppression of T-cell reconstitution is greatest at three weeks and still evident at four weeks post PBL injection.

Figure 4 is a graph which plots the average percentage of CD4, CD8 and $CD45^+$ positive cells in the peripheral circulatory system and in the spleen against time expired since PBL injection (Im Tx- OKT-3 restrictocin treated SCID mice, NC- negative control PBS treated mice.) The data for the first two time

points is identical to the data presented in Figure 3. These data demonstrate that after seven weeks the number of T cells in the spleens of OKT-3 restrictocin treated mice is greatly reduced as compared to control mice.

In summary, the single dose OKT-3 restrictocin treatment of PBL injected SCID mice produced a dramatic decrease in T cell numbers (about 50%). These results lead to the expectation that multiple dose treatments with OKT-3 restrictocin will decrease T cell numbers to a much greater extent. The desirablity of using OKT-3 restrictocin for the induction of immune tolerance is further demonstrated by the fact that no clinical toxicity was observed at the dose which produced the dramatic decrease in T cell numbers. The results indicate OCT-3 functions as an effector for delivery and selective uptake of restrictocin into targeted cells without appreciable uptake by unselected cells leading to toxicity. The inclusion of selective uptake is unknown and fortuitous.

INDUCTION OF IMMUNE TOLERANCE BY IMMUNOTOXIN

CLAIMS

What is claimed is:

1. A method of inducing immune tolerance to an allograft in a mammal comprising

administering a therapeutically effective amount of an immunotoxin consisting essentially of restrictocin molecularly conjugated to an antibody having OKT3-like binding specificity.

2. A method of inducing immune tolerance to histoincompatible transplanted tissue in a mammal comprising

administering restrictocin conjugated to an antibody having substantially the binding specificity of OKT3 in an amount effective to destroy essentially all T cells capable of mediating immune rejection of said transplanted tissue.

3. A method of inducing sustained tolerance of an allograft without continuous immunosuppression thereafter comprising

administering to a mammal restrictocin conjugated antibody having the T cell antigen recognition specificity of OKT3 in a dose sufficient to deplete all antiallograft reactive T cells, and

repopulating the immune system by natural regeneration of T cells in the absence of immunosuppressive agents.

4. A molecular conjugate useful for inducing permanent immune tolerance towards allogenic cells in a recipient animal comprising

restrictocin covalently conjugated to an antibody through a cleavable site, and

an antibody contained in said conjugate having OKT3 like characteristics.

5. The method of claims 1, 2, and 3 wherein said conjugated antibody is administered concurrently with a transplant procedure.

6. The method of claims 1, 2, and 3 wherein said conjugated antibody is administered at any time after a transplant procedure.

7. An article of manufacture comprising

a sterile container of the type used in the pharmaceutical art to contain an injectable solution

a solution contained in said container comprising a restrictocin conjugated antibody having the T cell antigen recognition specificity of OKT3, and

a label affixed to said container bearing directions for administration of said conjugated antibody to a mammal in a dose sufficient to destroy essentially all T cells having allograft rejection capability.

8. The method of claims 1 to 5 wherein said restrictocin conjugated antibody is conjugated through an unprotected sulfhydryl group.

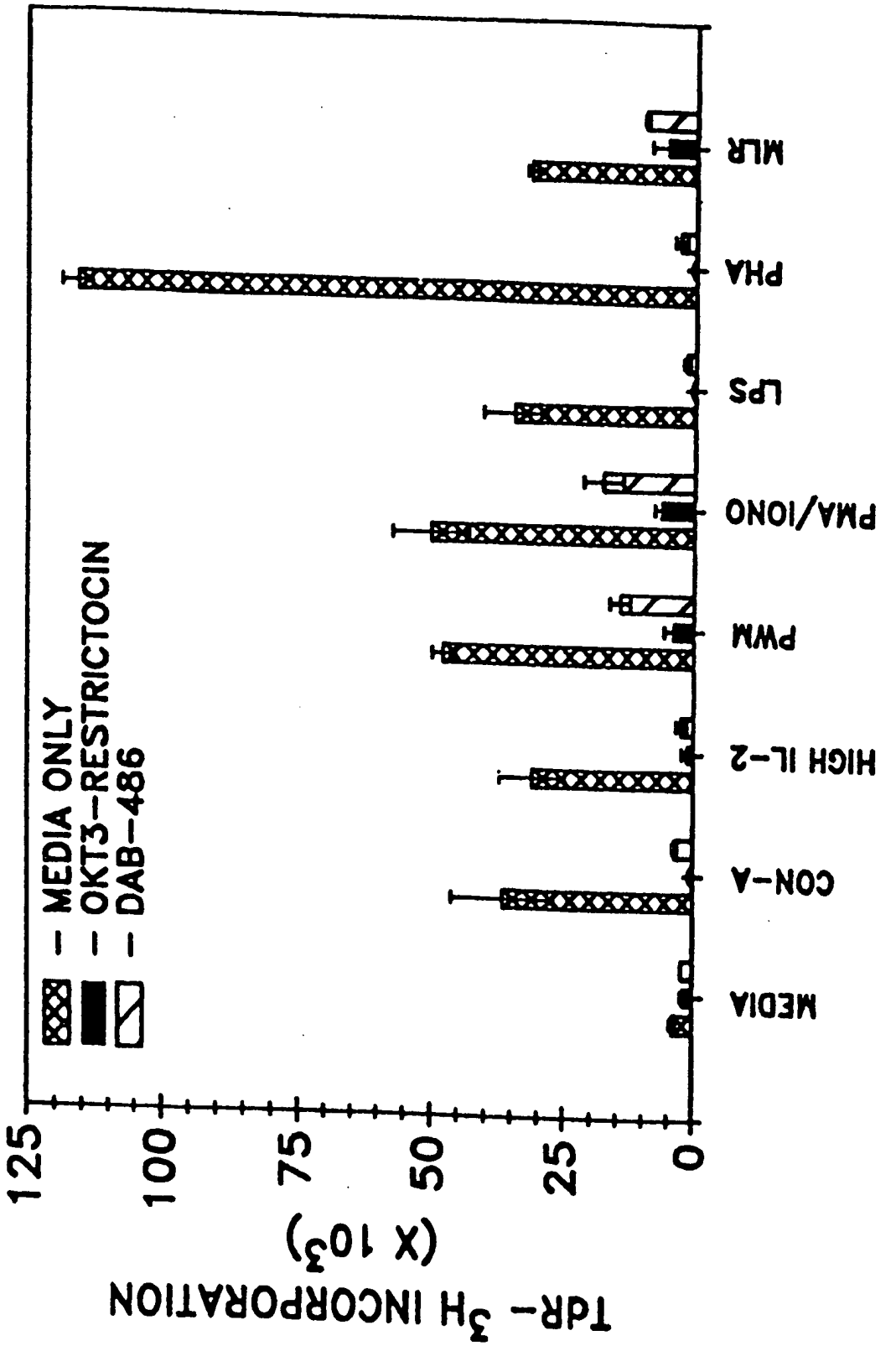
TdR- ^{3}H INCORPORATION
(X 10^3)
125
100
75
50
25
0
MEDIA
CON-A
HIGH IL-2
PWM
PMA/IONO
LPS
PHA
MLR
- MEDIA ONLY
- OKT3-RESTRICTOCIN
- DAB-486

Figure 1

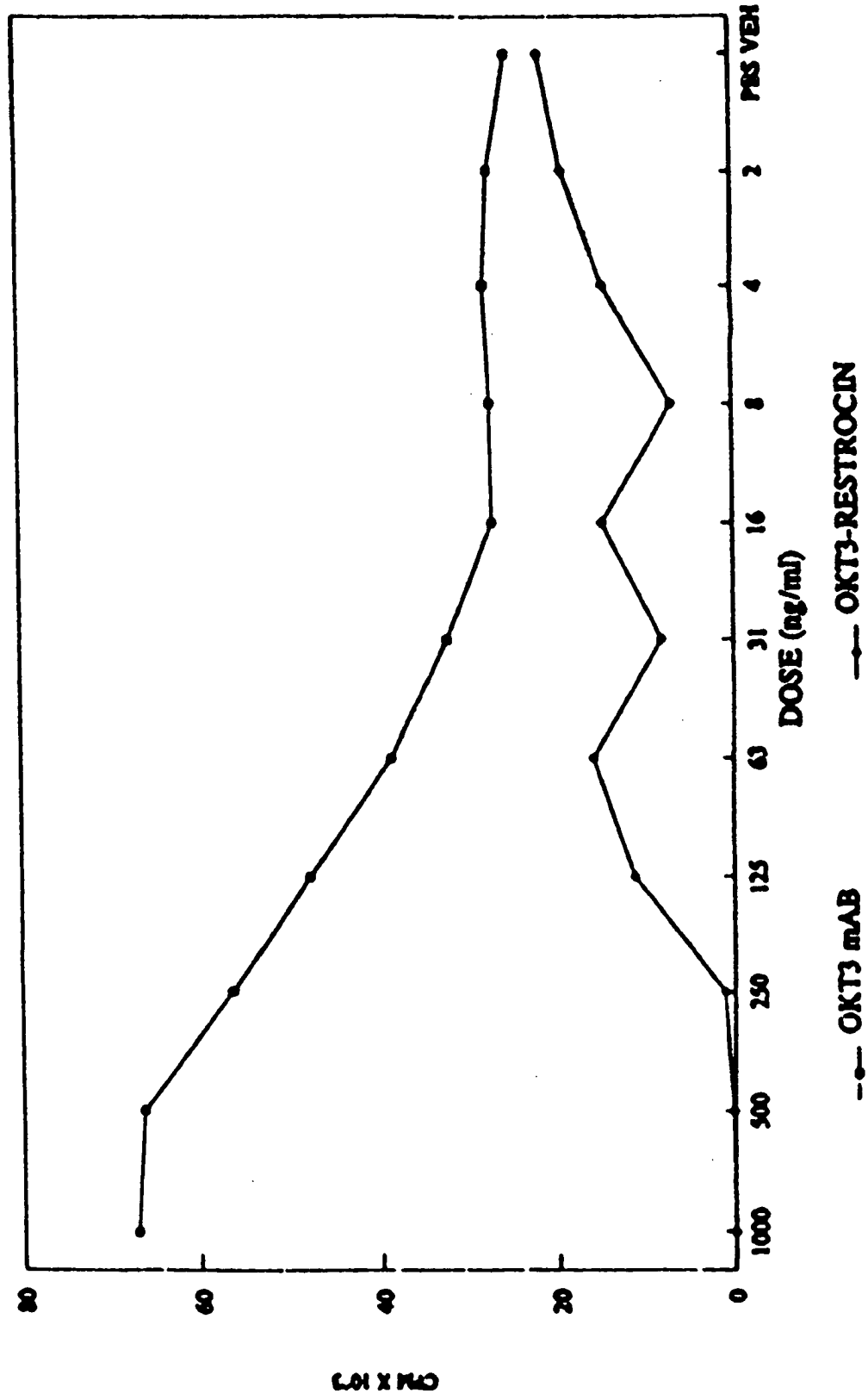
PBS VEH
2
4
8
16
31
63
125
250
500
1000
DOSE (ng/ml)
OKT3-RESTROCIN
OKT3 mAB
0
20
40
60
80

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11963

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/28; A61K 39/395, 39/40, 39/42, 38/44

US CL : 530/391.7; 424/178.1, 179.1, 180.1, 181.1, 182.1, 183.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/391.7; 424/178.1, 179.1, 180.1, 181.1, 182.1, 183.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,629,194 A (DINSMORE) 13 May 1997, see entire document, especially column 15, lines 16-41 (note the recitation of OKT3 at line 30), column 8, line 53, and column 14, line 53 to column 15, line 65.	1-7
Y	RATHORE et al. Generation of Active Immunotoxins Containing Recombinant Restrictocin. Biochemical and Biophysical Research Communications. 06 May 1996, Vol. 222, No. 1, pages 58-63, see entire document, especially the abstract and page 59 first paragraph.	1-7
A	US 5,066490 A (NEVILLE Jr. et al) 19 November 1991, see entire document.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 JULY 1998	Date of mailing of the international search report 03 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOSEPH W. RICIGLIANO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11963

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,725,857 A (NEVILLE et al) 10 March 1998, see entire document.	1-7
A	BYERS et al. Use of an Anti-Pan T-Lymphocyte Ricin A Chain Immunotoxin in Steroid-Resistant Acute Graft-Versus-Host Disease. Blood. 01 April 1990, Vol. 75, No.7, pages 1426-1432, see entire document.	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11963

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.